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## REVIEW

# The role of beta cell heterogeneity in islet function and insulin release

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## Abstract

It is becoming increasingly apparent that not all insulin-secreting beta cells are equal. Subtle differences exist at the transcriptomic and protein expression levels, with repercussions for beta cell survival/proliferation, calcium signalling and insulin release. Notably, beta cell heterogeneity displays plasticity during development, metabolic stress and type 2 diabetes mellitus (T2DM). Thus, heterogeneity or lack thereof may be an important contributor to beta cell failure during T2DM in both rodents and humans. The present review will discuss the molecular and cellular features of beta cell heterogeneity at both the single-cell and islet level, explore how this influences islet function and insulin release and look into the alterations that may occur during obesity and T2DM.

## Key Words

- ▶ insulin secretion
- ▶ diabetes II
- ▶ metabolism
- ▶ pancreatic beta cell

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## Introduction

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder which occurs when genetically susceptible individuals are exposed to a permissive environment. It currently affects ~10% of the adult population (Ezzati & World Health O 2004) and represents an escalating healthcare crisis. T2DM not only shortens lifespan and decreases quality of life, but also consumes healthcare budgets, with global costs approaching £825 billion per year (2016). During the early stages of T2DM, peripheral (and possibly central (Kullmann *et al.* 2016)) insulin resistance is compensated by an increase in insulin secretion from the beta cell mass, maintaining glucose homeostasis (Samuel & Shulman 2012). However, at a poorly defined point in time, beta cells are pushed over the functional precipice, resulting in relative insulin insufficiency and a large ramp-up in circulating glucose concentration (Halban *et al.* 2014). Together with

dyslipidaemia, hyperglycaemia drives the wide-ranging sequelae of T2DM including retinopathy, neuropathy, vasculopathy, renal and cardiovascular disease and increased risk of developing cancer (Forbes & Cooper 2013). In addition to diet and exercise, T2DM treatment relies on the lifelong use of oral anti-diabetic agents, which boost insulin release, improve insulin action or increase renal glucose excretion (Fowler 2007). However, a significant number of patients will eventually transition to exogenous insulin injection, probably reflecting the loss of any residual beta cell function (Home *et al.* 2014).

While current T2DM treatment aims to preserve endogenous insulin release for as long as possible, prevention of beta cell failure remains an important goal. The mechanisms through which single beta cells fail are well defined, at least in rodents, and encompass loss of glucose responsiveness, de-differentiation,

ER stress and apoptosis, all probably secondary to a combination of glucolipotoxicity and cytotoxicity (Porte & Kahn 2001, Prentki & Nolan 2006). Maintenance of insulin release during T2DM therefore requires better understanding of the mechanisms underlying beta cell proliferation and function in adults, with the hope that these can be harnessed or maintained to restore insulin secretion. Recent studies have opened up new avenues in our investigation of beta cell failure and insulin secretion, by showing the existence of subtle molecular and cellular differences between beta cells which give rise to proliferative and functional subpopulations (Gutierrez *et al.* 2017). This 'heterogeneity' or 'diversity' is driven partly by the organisation of beta cells into islet microorgans (Benninger & Piston 2014) and is unlikely to be properly appreciated by current models of beta cell failure. Importantly, beta cell subpopulations may contribute to development and proliferation of other beta cells, as well as play an exaggerated role in insulin secretion (Roscioni *et al.* 2016, Avrahami *et al.* 2017, Carrano *et al.* 2017). Thus, beta cell heterogeneity or diversity should be an important consideration when attempting to understand T2DM pathogenesis, since subpopulations may be differentially targeted by insults or treatments. Conversely, islet-like structures engineered from induced pluripotent stem cells for transplantation, and even genome editing, may need to take into account beta cell heterogeneity.

The present review will discuss the influence of beta cell heterogeneity on islet function and insulin release, and its relevance for T2DM. Particular attention will be paid to recent single-cell studies describing some of the molecular and cellular underpinnings of beta cell heterogeneity. We will also focus on the role of the islet context in giving rise to beta cell heterogeneity by discussing *in situ* and *in vivo* imaging studies. Finally, new technologies available for the precise interrogation of beta cell heterogeneity will be described, before highlighting future challenges for the field, including translation of results to the clinic.

### Stimulus–secretion coupling in single beta cells

Beta cells are well adapted as glucose sensors. Due to expression of low-affinity glucose transporters (GLUT1 in humans, GLUT2 in rodents) and glucokinase (German 1993, De Vos *et al.* 1995), beta cells only respond to elevated glucose, avoiding the inappropriate and damaging release

of insulin. Following transport into the cytosol, the sugar stimulates oxidative and glycolytic metabolism, leading to ATP generation in the mitochondria at the expense of other pathways (e.g. pentose phosphate shunt). The ensuing increase in cytosolic ATP/ADP ratio closes ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels, reducing K<sup>+</sup> efflux through the Kir6.2 pore and depolarising the membrane (Ashcroft *et al.* 1984). Together with Na<sup>+</sup> conductance, this drives action potential firing and the opening of voltage-dependent Ca<sup>2+</sup> channels (VDCC): primarily L-type in rodents but T-, P/Q- and L-type in humans (Rorsman & Braun 2013, Rutter & Hodson 2013). The increased cytosolic Ca<sup>2+</sup> fluxes interact with small N-ethylmaleimide-sensitive factor receptor proteins such as vesicle-associated membrane protein, synaptosomal-associated protein of 25 kDa (SNAP25) and syntaxin (Wheeler *et al.* 1996, Kwan & Gaisano 2009), stimulating insulin granule translocation to the membrane, granule fusion and dissolution of insulin crystals into the circulation. Insulin secretion is further assisted by 'amplifying' pathways (e.g. cAMP), which are K<sub>ATP</sub> channel independent but beta cell metabolism-dependent (Henquin 2000). Indeed, molecules such as hormones, neurotransmitters, nucleotides, amino acids and lipids are all able to strongly influence insulin secretion by either acting as nutrients or through interactions with receptors/ion channels. Perhaps the best characterised signals are derived from the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) released from the enteroendocrine L-cells and K-cells, respectively, following food intake and bile acid transit (Diakogiannaki *et al.* 2012, Parker *et al.* 2012). GIP and GLP-1 bind to their cognate G-protein-coupled receptors (GPCRs) on beta cells, stimulating adenylate cyclase activity and cAMP generation, activation of PKA and Epac, as well as ERK and beta arrestins (Baggio & Drucker 2007, Leech *et al.* 2011, Campbell & Drucker 2013). Through interactions with VDCC and the exocytotic machinery (MacDonald *et al.* 2002, Gromada *et al.* 2004, Leech *et al.* 2011), GIP and GLP-1 boost insulin secretion (so-called 'incretin effect'), largely accounting for the extra portion of the hormone secreted in response to oral vs intravenous glucose (Nauck *et al.* 1986, Nauck & Meier 2016). Since GIP receptors are downregulated/desensitised during obesity/T2DM (Lynn *et al.* 2001, Meier & Nauck 2010), stabilised variants of GLP-1 have served as a template for production of incretin mimetics. For an in-depth review of stimulus–secretion coupling in the beta cell, the reader is referred to (Rutter *et al.* 2015).

## Single-cell studies of beta cell heterogeneity

The concept of heterogeneity is not new. Differences in insulin release (Salomon & Meda 1986), glucose metabolism (Kiekens *et al.* 1992), glucokinase (Jetton & Magnuson 1992), insulin expression (Schuit *et al.* 1988), membrane potential (Dean & Matthews 1968) and  $\text{Ca}^{2+}$  (Zhang *et al.* 2003, Kenty & Melton 2015), cAMP (Dyachok *et al.* 2006) and NAD(P)H (Piston *et al.* 1999) signals have all been described in beta cells. However, it is only over the past decade that advances in imaging, genomics and proteomics have allowed a more detailed snapshot of the molecular and cellular drivers of beta cell heterogeneity. A spate of single-cell screening studies (See 1 in Table 1) in mouse and human islets have segregated out a number of beta cell subpopulations according to their transcriptomic profile, for example, due to differing abundances of genes for ER/oxidative stress (Baron *et al.* 2016, Muraro *et al.* 2016), and in the process have provided useful beta cell ‘atlases’ (see Supplementary methods for a summary of single-cell screening methodology, as well as the section on supplementary data given at the end of this article). However, relatively few studies have investigated subpopulation plasticity and its potential to influence disease or islet function. Using marker analysis coupled with single-cell RNA sequencing (scRNA-seq), Dorrell *et al.* were able to show the existence of four distinct human beta cell subpopulations ( $\beta 1$ –4), based upon differing expression of ST8SIA1 and CD9. Notably, the ST8SIA1-positive  $\beta 3$  and  $\beta 4$  populations exhibited lower insulin release during T2DM (Dorrell *et al.* 2016). At the protein level, high-throughput analyses of human beta cells immunostained with metal-labelled antibodies and subjected to time-of-flight mass cytometry (CyTOF) (See 2 in Table 1) showed three states: C1, C2 and C3 (Wang *et al.* 2016a). The C1 state – shown to be  $\text{PDX1}^{\text{high}}/\text{Ins}^{\text{high}}$  and poorly proliferative – was plastic depending on age and obesity, and increased in incidence with time (Wang *et al.* 2016a). This suggests that, during ageing and metabolic stress, there is a shift from a proliferative to a more mature beta cell phenotype, which may partly explain the limited capacity to regenerate beta cells in the adult, as well as their eventual failure under chronic metabolic burden.

Analysis of cell surface markers revealed that the Wnt/planar polarity effector, Fltp or Flattop, demarcates proliferative and mature beta cells (Bader *et al.* 2016). Fltp<sup>+</sup> beta cells are more mature, with improved insulin secretion and mitochondrial function, whereas the Fltp<sup>−</sup> beta cell subpopulation expands during metabolic stress, displaying increased proliferation. Thus, an increase in

the ratio of Fltp<sup>−</sup>/Fltp<sup>+</sup> cells may be expected to promote/maintain beta cell mass during T2DM, but would be expected to compromise insulin release due to their relatively poor glucose responsiveness. Along similar lines, Van der Meulen *et al.* identified a rare (~2% proportion) subpopulation of beta cells, characterised by the absence of urocortin 3 (Ucn3) expression, which represent an intermediate stage during the transdifferentiation of alpha to beta cells, thus acting as a ‘neogenic niche’ (van der Meulen *et al.* 2017). Ucn3<sup>−</sup> cells were metabolically naive (i.e. low glucokinase and oxidative phosphorylation) and were unable to properly sense glucose or support  $\text{Ca}^{2+}$  fluxes in response to glucose (van der Meulen *et al.* 2017), supporting previous observations that functional beta cell maturation is associated with increased Ucn3 expression (Blum *et al.* 2012). See Table 2 for a summary of selected beta cell subpopulations involved in islet plasticity and insulin release. The reader is referred to (Roscioni *et al.* 2016, Avrahami *et al.* 2017, Carrano *et al.* 2017, Gutierrez *et al.* 2017) for more comprehensive reviews of subpopulations identified to date.

Together, high-throughput scRNA-seq, CyTOF or reporter studies have provided unexpected insights into beta cell heterogeneity, identifying a number of subpopulations that display high degree of plasticity. The binary nature of these subpopulations appears to be a common trait: subgroups with two broad characteristics predominate during metabolic stress and ageing. Beta cells with decreased maturity, glucose sensing, metabolism and insulin secretion increase in proportion during metabolic stress, which may reduce islet function at the expense of maintaining beta cell renewal. Nevertheless, this may be balanced by expansion of beta cells with increased maturity and insulin expression, but poorer proliferative capacity. Whether these phenotypic shifts are interdependent is not known, but this raises the tantalising prospect that the islet is geared toward preserving heterogeneity and that eventual loss of this (i.e. as a subpopulation begins to predominate) is a key trigger for insulin secretory failure.

There are a number of caveats that need to be carefully considered when interpreting transcriptomic and biomarker-based studies. RNA-seq is limited in its coverage of the genome, with ~10% genes being missed even at high read numbers (Anisimova *et al.* 2015). While CyTOF resolution is excellent compared to conventional fluorescence-activated cell sorting (FACS) (Giesen *et al.* 2014), the poor availability of metal-labelled antibodies for many markers means that screening is still limited/partially biased, although this is expected to improve with labelling kits. In marker-based studies, reporter

**Table 1** Summary of technologies available for interrogating beta cell heterogeneity.

Technology	Advantages	Disadvantages	Reference
RNA-seq (1)	<ul style="list-style-type: none"> <li>• Transcriptome-wide coverage</li> <li>• Sensitive – requires ng of DNA</li> </ul>	<ul style="list-style-type: none"> <li>• Islet dissociation required</li> <li>• Cells with upregulated stress pathways generally excluded from analysis</li> <li>• Still expensive</li> <li>• Biological and technical variability due to (1) loss of transcripts during RNA isolation or (2) transcript coverage</li> </ul>	<a href="#">Ozsolak &amp; Milos (2011)</a>
CyTOF (2)	<ul style="list-style-type: none"> <li>• Parallel screening of up to 40 markers</li> <li>• Three orders of magnitude between detection channels</li> <li>• Excellent discrimination of negative and positive populations</li> <li>• Good discrimination of high-low-negative populations</li> </ul>	<ul style="list-style-type: none"> <li>• Islet dissociation required</li> <li>• Expensive machinery required</li> <li>• Antibody panels available, but limited selection for metabolism</li> <li>• Antibodies need to be labelled in house if not available</li> <li>• General lack of reliable antibodies for some beta cell proteins/markers</li> </ul>	<a href="#">Proserpio &amp; Lönnberg (2016)</a>
Optogenetics (3)	<ul style="list-style-type: none"> <li>• Conditionally targeted</li> <li>• Well characterised</li> <li>• Spectral variants</li> <li>• Stimulatory or inhibitory variants</li> </ul>	<ul style="list-style-type: none"> <li>• Presence of a non-mammalian channel/pump may affect cell function</li> <li>• Protein overexpression may interfere with cell function</li> <li>• Limited use in human tissue</li> </ul>	<a href="#">Johnston et al. (2016)</a>
Photopharmacology (4)	<ul style="list-style-type: none"> <li>• Usually based on known drugs/molecules</li> <li>• No need for genetic introduction</li> <li>• Exogenously applied</li> <li>• Can be spectrally-tuned</li> <li>• Useful in human tissue</li> <li>• Not conditional</li> </ul>	<ul style="list-style-type: none"> <li>• Non-binary response (i.e. generally some activity)</li> <li>• Can undergo metabolism depending on molecule</li> <li>• Subject to normal pharmacokinetics/pharmacodynamics</li> <li>• UV light is required for photoswitching of the majority of available compounds</li> </ul>	<a href="#">Broichhagen et al. (2015c)</a>
Tethered pharmacology (5)	<ul style="list-style-type: none"> <li>• Usually based on known drugs/molecules</li> <li>• Conditionally targeted using enzyme self-labels or engineered cysteine residues.</li> <li>• Can be combined with photopharmacology to make tethered photoswitches</li> </ul>	<ul style="list-style-type: none"> <li>• Use in human and mouse primary tissue requires viral vectors</li> <li>• Protein overexpression may interfere with cell function</li> </ul>	<a href="#">Podewin et al. (2018)</a>
Single-cell metabolomics (6)	<ul style="list-style-type: none"> <li>• Dynamic snapshot of cell metabolism</li> <li>• Can be performed in the intact tissue setting</li> </ul>	<ul style="list-style-type: none"> <li>• Slow throughput compared to CyTOF and RNA-seq</li> <li>• Sensitivity still relatively poor compared to conventional metabolomics</li> </ul>	<a href="#">Aerts et al. (2014), Ibanez et al. (2013)</a>
Transcriptome <i>in vivo</i> analysis (7)	<ul style="list-style-type: none"> <li>• Can be performed in the intact tissue</li> </ul>	<ul style="list-style-type: none"> <li>• Requires cell-penetrating peptides to introduce the TIVA-tag</li> <li>• Slow throughput</li> </ul>	<a href="#">Lovatt et al. (2014)</a>
<i>In vivo</i> imaging (8)	<ul style="list-style-type: none"> <li>• Allows investigation of beta cell function with preserved blood and neural supplies</li> </ul>	<ul style="list-style-type: none"> <li>• Technically challenging</li> <li>• Needs expensive microscopy equipment</li> <li>• Slow throughput</li> </ul>	<a href="#">Speier et al. (2008b)</a>



**Table 2** Selected beta cell subpopulations involved in islet plasticity and insulin release.

Subpopulation	Features	Plasticity	Reference
ST8SIA1 <sup>-</sup>	GLUT2+++ Insulin+++ Maturity+++	↓ T2DM	<a href="#">Dorrell et al. (2016)</a>
ST8SIA1 <sup>+</sup>	GLUT2+ Insulin+ Maturity+	↑ T2DM	<a href="#">Dorrell et al. (2016)</a>
C1	Insulin+++ Maturity+++	↑ Ageing ↓ Obesity	<a href="#">Wang et al. (2016a)</a>
Fltp <sup>+</sup>	Metabolism+++ Insulin+++ Maturity+++ Proliferation+	↓ Metabolic stress ↓ Metabolic stress	<a href="#">Bader et al. (2016)</a>
Fltp <sup>-</sup>	Metabolism+ Insulin+ Maturity+ Proliferation+++	↑ Metabolic stress ↑ Metabolic stress	<a href="#">Bader et al. (2016)</a>
Ucn3 <sup>-</sup>	Metabolism+ Ca <sup>2+</sup> + Maturity+ Transdifferentiation+++	↑ Metabolic stress	<a href="#">van der Meulen et al. (2017)</a>
Hub (eNpHR3.0)	Metabolism+++ Ca <sup>2+</sup> +++ Insulin++ Maturity++	↓ Metabolic stress	<a href="#">Johnston et al. (2016)</a>
Chr2	Metabolism+++ Ca <sup>2+</sup> +++	↓ Metabolic stress	<a href="#">Westacott et al. (2017b)</a>

constructs may not faithfully report protein expression, and in many cases, display limited changes in expression vs the protein under examination. Furthermore, how the subpopulations described to date co-exist together in the islet is not well understood, and this may be complicated by the relative dynamics of subpopulation plasticity. So far, most studies report results obtained at a specific time point (e.g. neonatal, postnatal and adult), without taking into account the changes that may appear in-between the time of assessment. Lastly, following dissociation from the islet, beta cells may lose many characteristics endowed through cell–cell contacts/signalling, certain stress pathways may be upregulated and a study may be skewed toward subpopulations that are more robust (*hint*: some are fragile, see below).

### Functional beta cell heterogeneity in the intact islet

In response to glucose, beta cells display intense Ca<sup>2+</sup> oscillations, which are not completely synchronous but

well-coordinated throughout the syncytium ([Benninger et al. 2008](#), [Stozer et al. 2013](#)). By contrast, beta cells cultured in two dimensions mount much more stochastic responses to secretagogues, release less insulin *per capita* and possess decreased insulin biosynthetic capacity ([Lernmark 1974](#), [Hodson et al. 2013](#)). This diminished functionality develops as a result of the loss of three-dimensional electrical communications through gap junctions following islet dissociation. Indeed, gap junctions couple on average 6–7 neighbouring beta cells, with a speed of electrical exchanges of ~13.5 ms, commensurate with the transmission of Ca<sup>2+</sup> signals (~80 μm/s) ([Aslanidi et al. 2001](#), [Benninger et al. 2008](#), [Zhang et al. 2008](#), [Stozer et al. 2013](#)). Studies using islets from mice globally or conditionally deleted for the gap junction protein connexin 36 (Cx36; encoded by *Gjd2*) – the major isoform expressed in beta cells ([Serre-Beinier et al. 2000](#)) – showed loss of coordinated responses to glucose, which resembled those observed in dissociated beta cells ([Ravier et al. 2005](#), [Head et al. 2012](#)). Other plausible mechanisms for beta cell–beta cell communications include diffusion of

chemical messengers (i.e. paracrine signalling) (Squires *et al.* 2002, Yang *et al.* 2011), contact-dependent signalling (i.e. ephrin) (Konstantinova *et al.* 2007) and ciliary (Gerdes *et al.* 2014) signalling, although the contribution of these slower communication modalities to islet function is less well characterised (reviewed in Rutter & Hodson 2014).

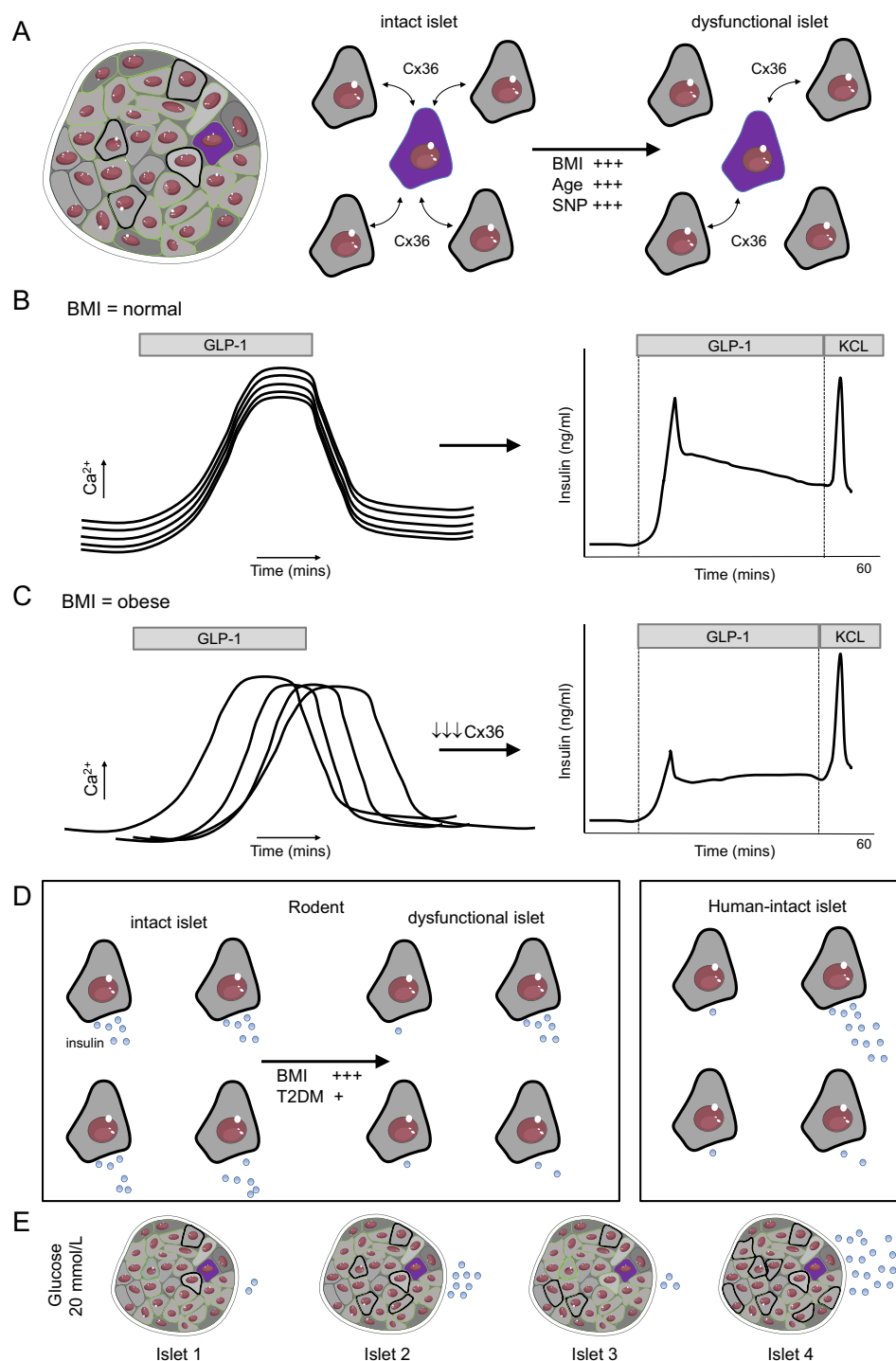
While human beta cells respond to glucose more stochastically (Martin & Soria 1996, Cabrera *et al.* 2006), regional coordination is detected between defined clusters (Cabrera *et al.* 2006, Westacott *et al.* 2017a), probably reflecting the differing architecture of islets in humans, where beta cell–beta cell interactions and gap junction signalling are constrained by an extra folding step during development (Bosco *et al.* 2010, Steiner *et al.* 2010). Human beta cells appear to be more dependent on the gut-derived peptide GLP-1 for coordinated responses. Incretin application induces large and synchronous  $\text{Ca}^{2+}$  rises that are lost following shRNA-mediated knockdown of *GJD2* or islet dissociation (Hodson *et al.* 2013). We speculate that the greater dependency on incretin input for coordination between human beta cells may reflect a mechanism to tightly entrain insulin release to mealtime, as GLP-1 is released from enteroendocrine L-cells following food ingestion (Diakogiannaki *et al.* 2012). By contrast, rodents graze during the dark phase, and in this scenario, glucose may provide adequate entrainment of insulin release with food intake. Further studies should look into the role of the enteroinsular axis in beta cell connectivity both in humans and rodents.

Coordinated beta cell–beta cell behaviour appears to be important for normal islet function and insulin release. Mice lacking *Gjd2/Cx36* show lowered indices of coordinated beta cell  $\text{Ca}^{2+}$  activity and loss of pulsatile insulin secretion both *in vitro* (Ravier *et al.* 2005) and *in vivo* (Head *et al.* 2012), leading to glucose intolerance despite preserved steady-state insulin release (Ravier *et al.* 2005, Head *et al.* 2012). Similarly, beta cell–beta cell coordination may be relevant for islet failure during T2DM. The beta cell population in islets from genetically obese *ob/ob* mice displays poorly organised responses to glucose with loss of pulsatile insulin release (Ravier *et al.* 2002), and *Cx36* expression and gap junction coupling strength between beta cells is markedly reduced by exposure to a pro-inflammatory cytokine cocktail (Farnsworth *et al.* 2015, Johnston *et al.* 2016). In human islets, coordinated beta cell responses to GLP-1 are inversely correlated with donor BMI and are lost following incubation with excess fatty acids, possibly due to a reduction in *Cx36* expression (Hodson *et al.* 2013) (Fig. 1A, B and C). A decline in beta cell coordination and gap junction coupling was also

associated with defective insulin secretory dynamics in islets from older donors (Westacott *et al.* 2017a). Lastly, knockdown of *ADCY5* and *Tcf7l2*, genes identified by GWAS as potentially harbouring single nucleotide polymorphisms (SNPs) increasing T2DM risk, led to marked decreases in beta cell–beta cell coordination (Hodson *et al.* 2014, Mitchell *et al.* 2014), as well as impaired glucose- and GLP-1-stimulated insulin secretion. Thus, loss of proper gap junction coupling and coordinated beta cell responses to secretagogues, may lead to impaired insulin release under conditions associated with T2DM (i.e. obesity, inflammation and gene silencing). For a comprehensive review of heterogeneity in the islet, see (Benninger & Piston 2014).

### Beta cell heterogeneity and insulin secretion

Differences in insulin secretion between individual beta cells were originally described using haemolytic plaque assay, including plasticity in response to pregnancy (Salomon & Meda 1986, Hiriart & Ramirez-Medeles 1991, Maedler *et al.* 2006). Subsequent studies using total internal reflection microscopy (TIRF-M) reported heterogeneity in secretory vesicle behaviour in rodent and human beta cells, including kiss-and-run exocytosis where fusion with the membrane occurs transiently, facilitating vesicle reuse (Tsuboi & Rutter 2003, MacDonald *et al.* 2006, Rutter & Hill 2006, Hanna *et al.* 2009), as well as differences in submembrane granule turnover and motility depending on stimulus and granule age (Hoboth *et al.* 2015, Bruning *et al.* 2017). However, TIRF-M only encompasses a dozen or so beta cells (Li *et al.* 2013), limiting the study of secretory heterogeneity across the entire islet. Nearly 20 years later, the advent of two-photon imaging enabled the tracking of the granule fusion with the beta cell membrane at nanometer axial resolution. Pioneering studies by Takahashi *et al.* deployed two-photon extracellular polar tracer imaging-based quantification (TEPIQ) to report uptake of the polar tracer, sulforhodamine B, into granules following fusion with the membrane, allowing exocytosis to be tracked via the appearance of fluorescent spots (Takahashi *et al.* 2002). Almost 60% of the beta cells were found to be involved in exocytosis, with the majority of events occurring toward the interstitial or non-vascular compartment. Interestingly, kiss-and-run exocytosis in the intact islet setting is an exceedingly rare event, suggesting heterogeneity is strongly shaped by cell–cell interactions (and possibly extracellular matrix content) (Takahashi *et al.* 2002, Ma *et al.* 2004). However, some polarity in insulin release was detected in intact islets, with

**Figure 1**

Beta cell communication and insulin release: (A) Gap junctions comprising connexin 36 allow beta cells to coordinate their activities within the intact islet. Gap junction signalling is reduced during obesity and ageing, as well as in individuals harbouring risk alleles for T2DM (SNP). (B) Beta cells in human islet mount coordinated responses to incretins such as glucagon-like peptide 1 (GLP-1), facilitating insulin release. (C) During obesity, a reduction in gap junction signalling leads to loss of coordinated beta cell activity, impairing GLP-1-stimulated insulin secretion. (D) Insulin secretion is polarised toward the vasculature and in rodent models of obesity and T2DM, a reduction in the number of actively secreting beta cells is detected. In humans, some beta cells and beta cell clusters contribute to insulin secretion more than others. (E) Islets display a large functional reserve, with only a handful of first responders supporting glucose-stimulated insulin secretion. Figures were adapted from Servier Medical Art under a CC-BY3.0 licence (<https://creativecommons.org/licenses/by/3.0/>).

exocytosis preferentially toward the vessels surrounded by beta cell rosettes (Takahashi *et al.* 2002). Similar TEPIQ studies showed that glucose recruits single cells throughout the islet to exocytosis, increases the number of fusion events per beta cell and induces coordinated oscillatory exocytotic activity, reminiscent of  $Ca^{2+}$  signals (Low *et al.* 2013) (Fig. 1D). The same group showed that, when viewed in three dimensions, insulin secretion is

asymmetric and targeted toward the vasculature (Fig. 1D), probably due to enrichment of liprin, piccolo and Rab2-interacting molecule at the vascular face (Low *et al.* 2014). Notably, beta cells in islets from glucose intolerant *db/db* mice showed some loss of insulin secretory heterogeneity, with 73% of cells becoming refractory to stimulation (Fig. 1D), although polarisation toward the vasculature was not studied (Do *et al.* 2014). In human islets, granule release



was shown to be pulsatile/oscillatory, with coordination detected only between beta cell clusters. The difference in coordination reflects the compartmentalisation of the  $\text{Ca}^{2+}$  response in this species, which could mirror the arrangement of beta cells vs alpha cells (Cabrerá *et al.* 2006, Almaca *et al.* 2015). In contrast to the mouse, a minority of beta cells contribute the majority of insulin secretion in humans, as assessed using haemolytic plaque assays (Wojtuszczyński *et al.* 2008) (Fig. 1D), although this may imply a prolonged secretion where secretory events tend to become more localised (Almaca *et al.* 2015). How heterogeneity in insulin secretion changes during T2DM is not well understood, probably due to difficulties in obtaining fresh tissue from donors. However, studies using matrix-assisted laser desorption/ionisation (MALDI) imaging in fixed tissue, together with *in vitro* experiments, demonstrated that stearyl carnitine accumulated in beta cells to arrest insulin synthesis, while acetyl carnitine and N-acyl taurines increased insulin secretion to induce beta cell failure (Aichler *et al.* 2017).

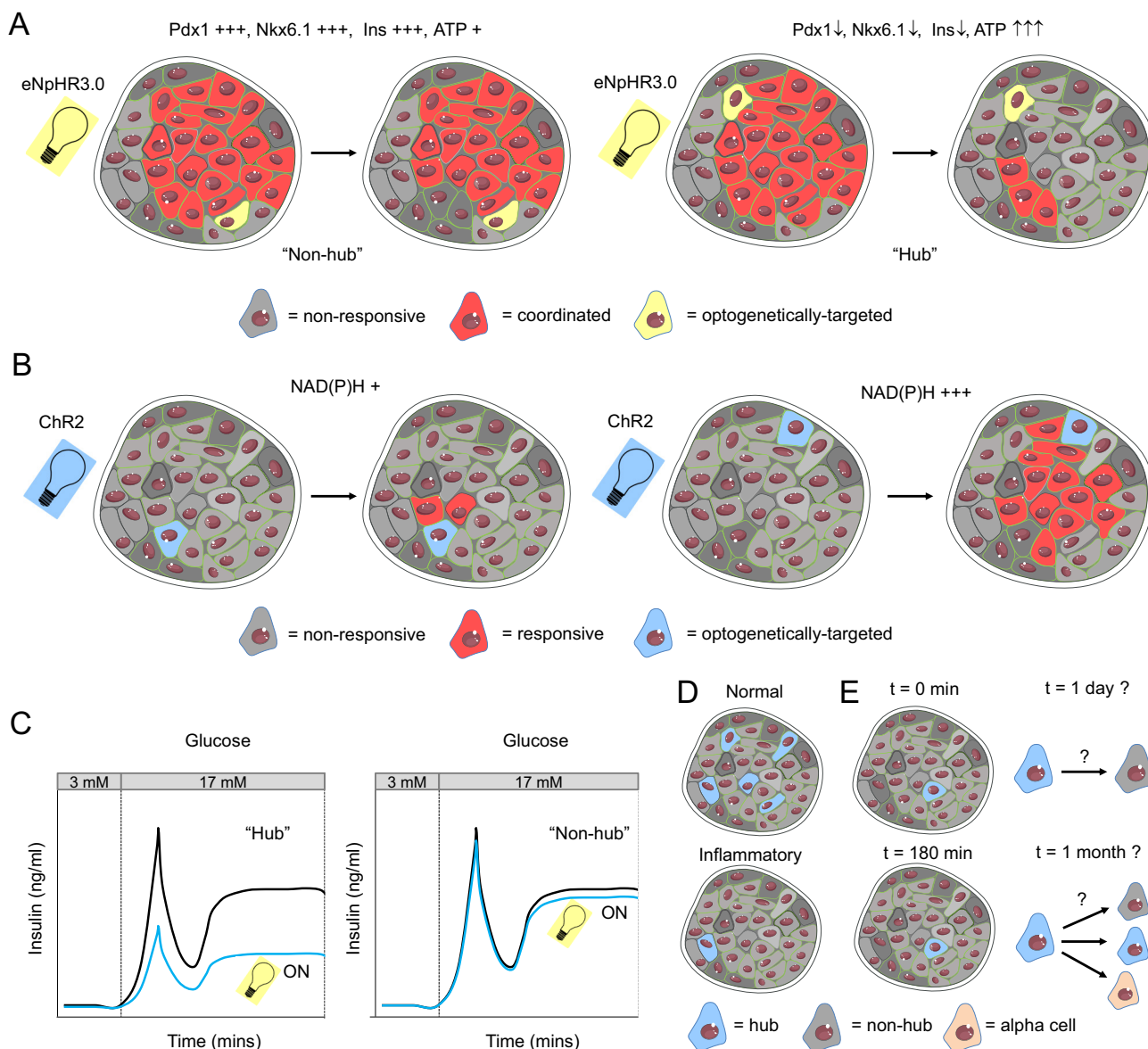
By contrast to *in vitro* experiments, technically demanding live imaging of the pancreatic surface in anaesthetised animals revealed that only a fraction of islets responded to acute glucose administration (Fig. 1E). This was characterised by rapid dispossession of insulin, transgenically marked using C-peptide-bearing superfolder GFP (Zhu *et al.* 2016). While the contribution of individual beta cells was likely not determined at the resolutions employed in these studies, it is nonetheless intriguing that most islets are unresponsive, suggestive of a functional reserve and tertiary level of heterogeneity (Zhu *et al.* 2016). Thus, significant heterogeneity in insulin secretion exists *in vitro*, with recruitment, coordination and polarisation toward the vasculature, with species differences appearing to reflect  $\text{Ca}^{2+}$  patterning. Further studies are required to understand how this is sculpted *in vivo*, where neural and vascular supplies may also influence beta cell function, as well as the fate of secreted insulin depending on the metabolic status (Michau *et al.* 2015).

### Functionally interrogating beta cell heterogeneity *in situ*

Although coordinated,  $\text{Ca}^{2+}$  responses to glucose vary in space and time within the beta cell compartment. Some islet sub-regions respond early to stimulus (Stozer *et al.* 2013), with  $\text{Ca}^{2+}$  responses propagating as gap junction-dependent waves across the islet (Benninger *et al.* 2008, 2014), indicating a presence of so-called pacemakers

(Ämmälä *et al.* 1991, Squires *et al.* 2002, Benninger *et al.* 2014). Thus, beta cell–beta cell communication and  $\text{Ca}^{2+}$  signalling *in situ* are clearly heterogeneous. Optogenetics (See 3 in Table 1) allied to high-speed imaging has recently opened up the possibility to precisely interrogate the influence of beta cell heterogeneity directly in the intact islet, where endocrine cell interactions critical for proper insulin release are preserved. Studies by Reinbothe *et al.* and Kushibiki *et al.* employed channel rhodopsin 2 (ChR2), a light-activated  $\text{Na}^{+}$  channel, to optically control insulin release from beta cells *in vitro*, as well as *in vivo* in mice rendered diabetic with streptozotocin (Reinbothe *et al.* 2014, Kushibiki *et al.* 2015). Subsequent studies by our lab using the yellow/orange light-activated  $\text{Cl}^{-}$  pump halorhodopsin 3.0 (NpHR3.0) to pinpoint silence cell activity revealed that a minority of beta cells, termed hubs, are tasked with orchestrating islet-wide  $\text{Ca}^{2+}$  and insulin secretory responses to glucose (Johnston *et al.* 2016) (Fig. 2A and C). These beta cells constitute ~1–10% of the population at the islet surface, display activity profiles that precede and lead those of the rest of the population (i.e. pacemaker-like) and tend to host the majority of coordinated activity (*think*: network servers or major airports). To support their activity, hubs possess high levels of glucokinase and highly hyperpolarised mitochondria, indicative of increased ATP synthase activity and ATP generation. Unexpectedly, this was associated with lowered but not absent Pdx1 and Nkx6.1 and reduced insulin expression, resembling cells identified using RNA-seq ( $\text{GK}^{\text{high}}/\text{Pdx1}^{\text{low}}/\text{Nkx6.1}^{\text{low}}$ ) (Xin *et al.* 2016) and gene reporters ( $\text{Pdx1}^{+}/\text{insulin}^{\text{low}}$ ) (Szabat *et al.* 2009), and suggestive of a relatively immature phenotype.

How does metabolic adaptation in hubs fit with the apparent loss of beta cell identity, given that the latter supposedly endows the former? Maturity, as defined by expression of transcription factors such as Nkx6.1, Pdx1 and MafA may not always be obligatory for glucose metabolism. For example, NAD(P)H responses in beta cells deficient in MafA are almost normal (Hang *et al.* 2014) and deletion of a single *Pdx1* allele in beta cells increased apoptosis, but did not affect  $\text{Ca}^{2+}$  fluxes,  $\text{Ca}^{2+}$  conductance, glucose sensing or insulin secretion *in vitro* (Johnson *et al.* 2003). In addition, hubs may represent an immature and proliferative subpopulation in adults, similar to  $\text{Ucn3}^{-}$  cells (van der Meulen *et al.* 2017), although differentiated by their normal glucose responsiveness, heightened mitochondrial metabolism and improved  $\text{Ca}^{2+}$  fluxes. Indeed, replicating beta cells double RNA abundance of the majority of genes, except for those involved in key beta cell functions such as

**Figure 2**

Beta cell heterogeneity and islet function. (A) Optogenetic silencing of less mature and metabolically-adapted beta cells, termed hubs, leads to a loss of coordinated population activity (eNpHR3.0; halorhodopsin3.0, a yellow light-activated  $\text{Cl}^-$  pump). (B) Optogenetic activation of cells with high but not low NAD(P)H levels leads to activation across the islet (ChR2, channel rhodopsin 2, a blue light-activated  $\text{Na}^+$  channel). (C) Insulin secretion is impaired following silencing of hubs, but not other single beta cells. (D) Hubs are targeted by cytokines to mimic the pro-inflammatory milieu associated with T2DM. (E) Hubs are stable for a few hours, but their fate is not known over longer time periods. Possibilities include: (1) remaining a hub; (2) becoming a non-hub beta cell or (3) transdifferentiation into another cell type. Figures were adapted from Servier Medical Art under a CC-BY3.0 licence (<https://creativecommons.org/licenses/by/3.0/>).

secretion (Klochender *et al.* 2016). During postnatal development, immature proliferative cells possess high expression levels of amino acid metabolism and mitochondrial genes (Zeng *et al.* 2017). Nonetheless, hubs were susceptible to failure following introduction of a pro-inflammatory milieu, which may reflect a relative lack of Pdx1 and SERCA2, the former interacting with the latter to maintain organellar  $\text{Ca}^{2+}$  homeostasis and

protect against ER stress (Sachdeva *et al.* 2009, Johnson *et al.* 2014).

Using ChR2, Westcott *et al.* activated single beta cells at random and calculated the proportion of the islet showing corresponding  $\text{Ca}^{2+}$  elevations (Westcott *et al.* 2017b). While these elevations were restricted to closest neighbours in most experiments, in ~5% of trials  $\text{Ca}^{2+}$  spread over large islet regions. Suggesting metabolic

adaptation in these cases, the  $\text{Ca}^{2+}$  elevation was associated with cells/sub-regions displaying the highest NAD(P)H responses (Westacott *et al.* 2017b; Fig. 2B). However, the same authors showed that areas with low NAD(P)H and high  $\text{Ca}^{2+}$  oscillation frequency were associated with the origin of  $\text{Ca}^{2+}$  wave initiation. Whether these optogenetically defined beta cell subpopulations are distinct, or are overlapping but arise as a consequence of the wave propagation/correlation analysis used, needs to be further investigated. These studies together show that beta cell subpopulations *in situ* can play a disproportionate role in dictating islet responses to glucose, that this may be due to alterations in metabolism, and that these cells may fail in response to diabetes-like insults (Fig. 2A-E). It will be interesting to determine if these subpopulations have a similar protein barcode to hubs or other identified beta cell subpopulations that display metabolic adaptation.

Whether or not optogenetically defined beta cell subpopulations are stable or dynamic is difficult to assess due to restrictions on recording time *in vitro* (a few hours at most; Fig. 2E). Modelling studies have shown that pacemaker regions may stem from areas of highest excitability (Benninger *et al.* 2014), and this may change depending on ion channel expression levels (turnover times for Kir6.1/SUR1 complexes are estimated to be ~2.2h) (Crane & Aguilar-Bryan 2004) or proximity to non-beta cells (such as delta cells, shown to convey beta cell-driven alpha cell suppression) (Briant *et al.* 2017)). Moreover, how activity of single beta cells and small sub-regions can lead to  $\text{Ca}^{2+}$  signal propagation across large regions of the islet is not known. Presumably, gap junctions play a role, since their knockdown decreases the distance over which signals travel (Johnston *et al.* 2016) and disrupts beta cell–beta cell coordination (Ravier *et al.* 2005, Head *et al.* 2012). Other explanations may lie outside of the field of view, since most experiments only consider the top couple of islet layers. While 3D imaging of mouse islets is challenging due to the highly scattering nature of endocrine cells densely packed with secretory granules, studies in the more transparent zebrafish model using light sheet microscopy are likely to be more informative (Singh *et al.* 2017).

As a technology for interrogation of beta cell function, optogenetics is not without drawbacks. First, high (and potentially phototoxic) laser powers are required to activate ChR2 and especially eNpHR3.0, and constant light exposure can ground-deplete their activity cycle (i.e. render them refractory). Second,  $\text{Cl}^-$  and  $\text{H}^+$  pumps required for cell silencing can generate profound hyperpolarisation, similar to full  $\text{K}_{\text{ATP}}$  channel

opening, with yet unknown consequences on long-term beta cell function. The mechanisms occurring in beta cells likely involve transcriptional changes, given the recently reported interplay between  $\text{Ca}^{2+}$  influx and gene networks (Stancill *et al.* 2017). Third, ChR2 and eNpHR3.0 significantly improved glucose tolerance *in vivo* without illumination (Reinbothe *et al.* 2014, Johnston *et al.* 2016), an effect that cannot be explained by their activity, as no illumination was present. Lastly, all optogenes introduce an exogenous photocurrent, and while no issues have been reported for ChR2, an increase in beta cell excitability was reported following inactivation of eNpHR3.0 (Johnston *et al.* 2016), probably due to a collapse in the  $\text{Cl}^-$  gradient and depolarisation following a shift in the  $\text{Cl}^-$  reversal potential (Raimondo *et al.* 2012). Thus, as for RNA-seq, CyTOF and other techniques used in the study of beta cell heterogeneity, careful consideration of the datasets is required.

### Beta cell heterogeneity – more questions raised than answered?

Beta cell heterogeneity is highly plastic in response to a pro-diabetic milieu. Thus, ST8SIA1<sup>+</sup> beta cells increase in proportion in islets from T2DM donors (Dorrell *et al.* 2016), similar to Fltp<sup>+</sup> beta cells that expand in number following metabolic stress (Bader *et al.* 2016). Hubs, as well as gap junction signalling, fail in the face of inflammation (Farnsworth *et al.* 2015, Johnston *et al.* 2016), while beta cell ion channel number is altered during T2DM (Dorrell *et al.* 2016, Wang *et al.* 2016b), and exocytosis is restricted to only a few beta cells in islets from obese *db/db* mice (Low *et al.* 2014). However, the contribution of each event to T2DM and their inter-relations remain unclear. Are alterations in gap junction signalling preceded by subpopulation shifts to a more immature phenotype? If so, how can this be reconciled with the known molecular mechanisms underlying decreased gap junction coupling (i.e. protein kinase C delta) or evident heterogeneity in gap junction coupling strength (Farnsworth *et al.* 2015)? What is the overlap between the various subpopulations, given that mature Fltp<sup>+</sup> and ST8SIA1<sup>+</sup> cells both constitute ~80% of the beta cell population? What are the relative dynamics of subpopulation shifts (e.g. from ST8SIA1<sup>+</sup> → ST8SIA1<sup>+</sup>) and how does this relate to changes in function and insulin release? Are these shifts permanent or can they be reversed following treatment of T2DM or remission in mouse models? Key to answering some of these questions will be technologies that allow heterogeneity to be longitudinally tracked with high temporal resolution.

## New tools for understanding beta cell heterogeneity

The majority of recent understanding concerning beta cell heterogeneity has been derived from 'omics' or high-throughput approaches, such as RNA-seq and CyTOF. While these have identified subpopulations with similar characteristics in terms of maturity and insulin secretion, they have also highlighted differences in terms of the markers/genes that are differentially expressed. However, further description of heterogeneity will require the use of complementary techniques, as well as integration of the obtained datasets to increase detection sensitivity and accuracy.

### Photopharmacology

Photopharmacology, otherwise referred to as optochemistry, describes the use of light to control the cell's endogenous signalling apparatus/cytoskeleton (Velema *et al.* 2014). This approach relies on the synthesis of molecules whose inactive and active states can be precisely controlled by light. Central to photopharmacology, is the installation of photoresponsive elements, usually an azobenzene moiety, which undergoes isomerisation in response to illumination (a molecular motor) (Broichhagen *et al.* 2015c) (Fig. 3A) (See 4 in Table 1). The ensuing shift between *cis*- and *trans*-isomers subtly influences molecular motion, altering binding conformations and thus placing ion channel, receptor and enzyme function under optical control (Broichhagen *et al.* 2015c). A major advantage of photopharmacology over optogenetics is the lack of requirement for recombinant engineering: drugs can be exogenously applied, for example, light-activated sulfonylureas (Broichhagen *et al.* 2014, 2015b), incretins (Broichhagen *et al.* 2015d, 2016) and GPR40 agonists (Frank *et al.* 2017). Photopharmacology can serve as a tool to understand how GPCR or ion channel activation influences signalling and function in defined subpopulations, since these can feasibly be targeted with light as for hubs, with particular use in human islets. Pertinently, expression levels of *Trpm6*, and subunits for  $K_{ATP}$  and  $K^+$  channels all change in islets isolated from donors with T2DM (Dorrell *et al.* 2016, Wang *et al.* 2016b). However, there are disadvantages with photopharmacology: the lack of binary switch can complicate the optical control of cell function, UV illumination is generally required for the isomerisation step and toxicity and metabolite production need to be carefully assessed, just as for any drug,

although the azobenzene itself appears to be well tolerated (Mehta *et al.* 2017).

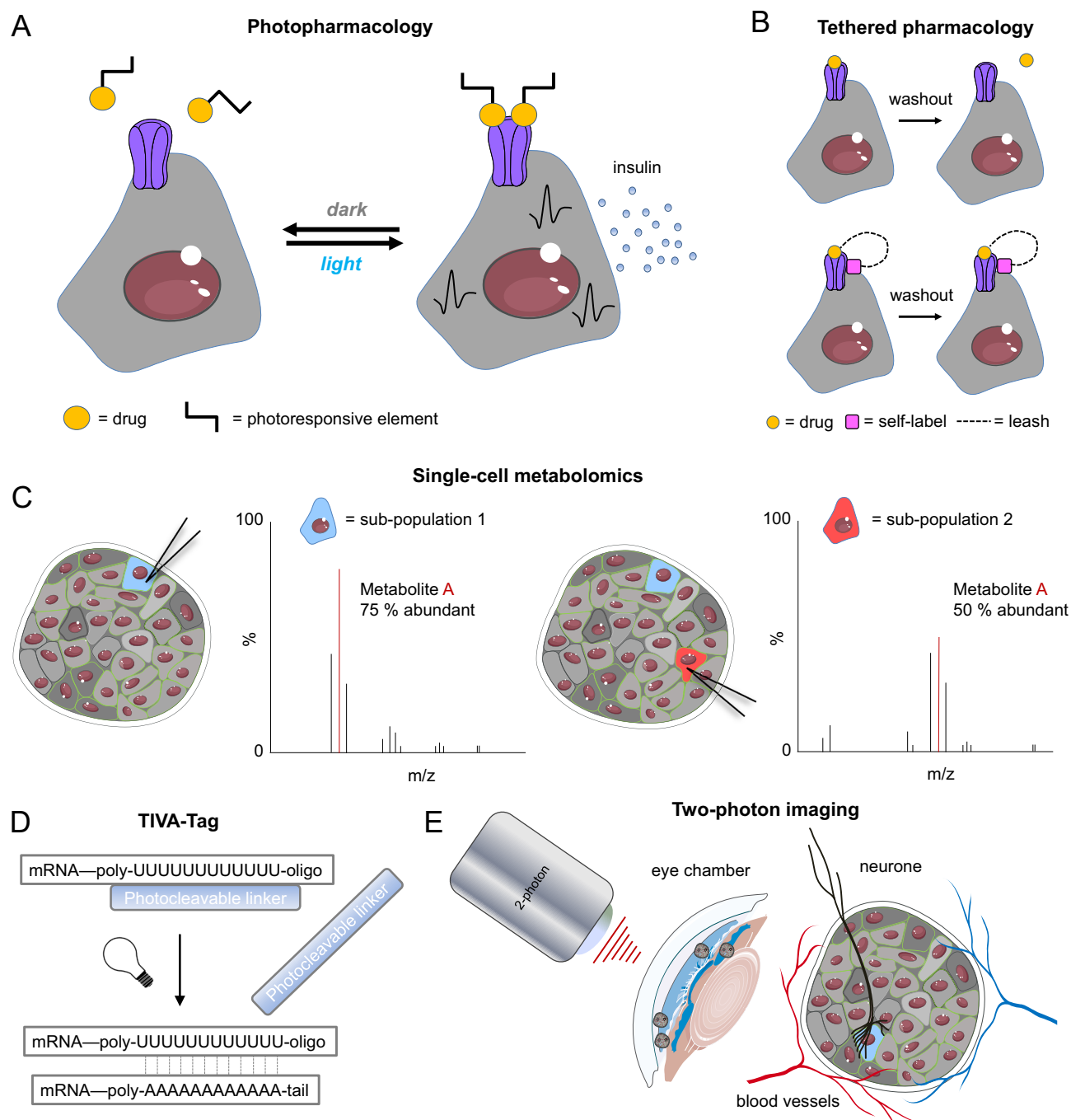
### Tethered pharmacology

Tethered pharmacology describes the production of small-molecule and peptide agonists that are restricted to the cell surface or to a specific receptor through protein self-labels (Leippe *et al.* 2017, Shields *et al.* 2017) (Fig. 3B) (See 5 in Table 1). Generally, this relies on covalent binding through SNAP tags, but can also take the form of engineered cysteines that react with maleimides introduced into the ligand (Leippe *et al.* 2017, Shields *et al.* 2017). Thus, tethered pharmacology allows cell signalling to be conditionally targeted, with the potential to further interrogate subpopulations using light-activated congeners, Cre-recombinase-directed expression of the SNAP-tag (Broichhagen *et al.* 2015a, Levitz *et al.* 2017) or even SNAP GPCR fusions, as recently shown for the GLP-1R in MIN6 beta cells (Podewin *et al.* 2018).

### Single-cell metabolomics

While single-cell RNA and protein sequencing approaches provide valuable information regarding a cell's potential, they are much less informative concerning dynamic responses to environment. By contrast, the metabolome gives a snapshot of metabolism by surveying the metabolites built up in a cell at a given time point (Johnson *et al.* 2016). Such an approach is clearly relevant for beta cells where oxidative and glycolytic metabolism underpin insulin release. However, investigating subpopulations is limited by the amount of tissue required for reliable metabolite measures, although metabolomics on FACS-enriched populations from multiple islets (e.g. of Fltp<sup>+</sup>, ST8SIA1<sup>+</sup> or Ucn3<sup>+</sup> cells) is plausible. Also, this does not take into account variability between single cells. Microarrays for mass spectrometry have been applied to single yeast cells, revealing co-existing subpopulations in isogenic populations (Ibanez *et al.* 2013). Along similar lines, single neurons were subjected to capillary electrophoresis-mass spectrometry and metabolomic analysis using cytoplasm extracted under direct visualisation (Fig. 3C) (See 6 in Table 1) (Aerts *et al.* 2014). While this is slow throughput, 3D holographic and tomographic laser microscopy coupled with a nanospray tip to extract picolitres of cytosol for mass spectroscopy may speed this up (Ali *et al.* 2016). Crucially, this circumvents the need to dissociate islets, meaning that metabolites can potentially be surveyed *in situ* in the whole islet.



**Figure 3**

Future technologies for interrogating beta cell heterogeneity. (A) Photopharmacology uses light-activated drugs to turn receptors and ion channels into endogenous photoswitches. (B) Tethered pharmacology combines the precision of genetics with pharmacology to selectively target specific cell and receptor populations, and even organelles. (C) Single-cell metabolomics subjects cytoplasm extracted from specific cells to capillary electrophoresis-mass spectrometry, potentially delineating differences in metabolite abundances between subpopulations. (D) TIVA-tag affords optical control over mRNA capture in single cells using a photocleavable poly-U-oligo, which hybridises the corresponding poly-A-tail. (E) Two-photon imaging of islets transplanted into the anterior chamber of the eye allows beta cell heterogeneity to be visualised in a setting where vascular and neural supplies re-wire. Figures were adapted from Servier Medical Art under a CC-BY3.0 licence (<https://creativecommons.org/licenses/by/3.0/>).



### Transcriptome *in vivo* analysis

Endocrine cells display stable gene expression patterns in a tissue context, with this becoming pulsatile following dissociation (Featherstone *et al.* 2011). Multiple rates of transcription can also be detected, with local coordination determined by gap junction communication (Featherstone *et al.* 2016). Such influence of the 3D architecture is likely to be lost following dissociation into single cells for high-throughput analysis, meaning heterogeneity may not be fully captured. Moreover, the unique extracellular microenvironment present *in vivo* may exert another level of heterogeneity on gene expression (Michau *et al.* 2015, Arzouni *et al.* 2017). Single-cell transcriptomes can be directly assessed in the tissue context directly *in vitro* and *in vivo* using transcriptome *in vivo* analysis (TIVA)-tags, which comprise a light-activated capture oligonucleotide that binds to the mRNA polyA tail, allowing affinity purification of TIVA-mRNA hybridised cells (Fig. 3D) (See 7 in Table 1) (Lovatt *et al.* 2014). Using this technique, it was possible to show that the *in vivo* tissue microenvironment drives bimodal gene expression (27 vs 645 bimodal transcripts in single neurones from culture compared to *in vivo*, respectively) (Lovatt *et al.* 2014).

### *In vivo* imaging

Little is known about *in vivo* function of beta cell subpopulations, other than static snapshots regarding their plasticity (e.g. proliferation of Fltp<sup>+</sup> cells or transdifferentiation of Ucn3<sup>+</sup> cells). The ability to study islets directly *in vivo* promises to provide novel information regarding the influence of the vascular or neural supplies on beta cell heterogeneity, whether heterogeneity contributes to insulin secretion *in vivo* and the dynamics of beta cell subpopulation plasticity (e.g. is it gradual or rapid? Do cells remain in a fixed state or interchange?). To facilitate such studies, rodent and human islets can be transplanted into the anterior chamber of the eye (Speier *et al.* 2008a,b), which provides an optically translucent window into beta cell function when combined with epifluorescent, confocal or multiphoton imaging and even optogenetics (Fig. 3E) (See 8 in Table 1). Moreover, neurons and vascular supplies re-wire (Rodriguez-Diaz *et al.* 2012, Diez *et al.* 2017), affording the opportunity to investigate the influence of these on beta cell heterogeneity, and function/fate/plasticity can be followed longitudinally in the same animal (e.g. in response to high fat diet or anti-diabetic drug therapy) (Abdulreda *et al.* 2016, Paschen *et al.* 2016). However, results need to be interpreted in light of some reported immune infiltration (Mojibian

*et al.* 2013) and lack of endocrine–exocrine interactions. By contrast, imaging of the pancreas via abdominal laparotomy allows investigation of endocrine–exocrine interactions and native blood and neural supplies (Nyman *et al.* 2008, 2010, Michau *et al.* 2015), but is complicated due to movement and is restricted predominantly to surface islets in the splenic region.

### Future challenges

Beta cell heterogeneity exists at many levels of islet operation, from genes > proteins > stimulus–secretion coupling > insulin release. High-throughput screening, as well as *in situ* imaging studies have provided unprecedented details regarding the molecular and cellular drivers of this heterogeneity, including subpopulations based upon their transcriptome, Ca<sup>2+</sup> responses and secretory capacity. However, how heterogeneity co-exists at multiple levels is poorly described, in particular, the overlap between subpopulations. Moreover, as many subpopulations identified to date are defined by multiple markers or gene patterns, it remains difficult to functionally interrogate their contribution to islet function using conditional silencing or overexpression approaches. Technical and bioinformatic constraints also limit the sensitivity to delineate subpopulations, especially in islets from T2DM donors where despite best efforts to age, sex and BMI-match, variability can still stem from drug therapy, historic glycaemic control and timing of diagnosis. Indeed, validation of genes differentially expressed in three separate single-cell studies using islets from normal and T2DM donors, revealed overlap between 54/77 and 32/171 significantly upregulated and downregulated genes, respectively (Lawlor *et al.* 2017). Nonetheless, this in itself demonstrates the power of combining RNA-seq results to finely resolve the transcriptome. We anticipate that the ever-increasing fidelity of single-cell technologies, the combination of functional and gene expression analysis and bioinformatics integration of datasets will expand the view of beta cell heterogeneity.

Going forwards, it will be important to demonstrate the relevance of heterogeneity for individuals with T2DM. Certainly, subpopulations with proliferative capacity could be harnessed to aid beta cell regeneration, although more detailed assessment of *in situ* functional heterogeneity is required in human islets to see if this reflects the available rodent data. Conversely, screens may identify drugs that protect susceptible subpopulations, preventing beta cell failure in the first place. The generation of beta cells from iPS cells should aim to recapitulate normal heterogeneity,

as changes in heterogeneity have consistently been shown to occur during T2DM, and in some cases, lead to lowered beta cell function. More broadly, the islet provides a tractable model for the study of heterogeneity in general, and results may be applicable to the study of other less accessible organs, for example, the pituitary gland where heterogeneity in at least two different axes has also been shown to be critical for proper hormone release (Sanchez-Cardenas *et al.* 2010, Hodson *et al.* 2012, Le Tissier *et al.* 2016).

#### Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-18-0011>.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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